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Study of optimal nutritional conditions for Arbutin production from *Bacillus subtilis* NN2M

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Abstract

Arbutin is a combination of D-glucose and hydroquinone. Among the positive effects of arbutin are its antioxidant properties, antimicrobial properties and anti-inflammatory properties. Arbutin is one of the most important active compounds used to inhibit the enzyme tyrosinase, which causes enzymatic browning in many foods. Microbial fermentation has emerged as an up-and-coming technique for the production of arbutin in recent years. The aim of the study was the biological production of arbutin from *Bacillus subtilis* NN2M isolates. The isolates were confirmed and identified by Vitek and *16S rRNA* and the nutritional conditions for production were improved to reach optimal conditions. To determine the best nutritional conditions for arbutin production, several conditions were studied, including the carbon source and its concentration, the nitrogen source and its concentration and the hydroquinone concentration. The results showed that the highest output of the active compound arbutin when using Molasses as a carbon source arbutin concentration was 23.34µg/mL. We also found the best concentration of the optimal carbon source, 4 % (w\v) arbutin concentration, was 27.62 µg/mL; furthermore, while using several nitrogen sources for producing arbutin, peptone was determined to be the optimum source, the concentration of arbutin was 47.3 µg/mL, the nitrogen source optimal Concentration was 2 % (w\v) arbutin concentration was 59.46 µg/mL, the optimum concentrations of hydroquinone was 2 % (w\v) arbutin concentration was 79.1 µg/mL.

Keywords

glycosides; arbutin; production bacterium; carbon source; nitrogen source

Introduction

The term "glycoside" encompasses a wide range of compounds derived from sugars and aglycone (non-sugary portion). Glycosides are molecules that have one or more sugar (glycone) moieties connected to them by a glycosidic bond. This connection connects the anomeric carbon of one glycone moiety to the functional group (s) of another glycone moiety. Phenolic compounds are typically present near the surface of green leaves or in the epidermal cells and they appear to shield leaves from the potential adverse effects of ambient UV light. Additionally, some studies have indicated that phenolic/flavonoid aglycones and glycosides are promising treatments (1). A monosaccharide like glucose, rhamnose, or fructose can make up the glycone part of the glycosides (2). Secondary metabolites with intriguing biological and pharmacological properties can be found in abundance in medicinal plants; glycosides are metabolites that are found

naturally and have exceptional. It is used as a nutritional supplement or alternative treatment in the management and treatment of cancer (3, 4). The presence of glycosides, phenols and flavonoids in the aqueous extract of mycelium *Ganoderma applonatum* and their effect on reducing cancerous tumors have been proven. One of the biological activities possessed by active compounds is their effect as antioxidants, which makes them among the promising anticancer compounds, including glycosides and flavonoids; this has been proven by (5). Arbutin is a phenolic glycoside. The presence of phenolic compounds is familiar in the plant kingdom. It exists as a compound secondary metabolism that plants produce in response to microbial diseases or insects (6). One everyday use of natural hydroquinone arbutin (4-hydroxyphenyl-b-D-glucopyranoside) is to stop the production of melanin. This is because it inhibits tyrosinase (7). The chemical formula of arbutin consists of a single molecule of D-glucose linked to hydroquinone. In water, D-glucose may be found in three different forms: α, β, and γ-anomer, the most common of which is β-anomer (8). Hydroquinone and the D-glucose anomer combine to form arbutin (9). At the same time, molasses was the best carbon source at a concentration of 5 % for producing kojic acid from local mould *Aspergillus oryzae* (10). Antioxidants are now widely utilized in pharmaceuticals, cosmetics, food packaging plastics, essential oils and processed foods; antioxidants are beneficial for more than just food preservation in addition to avoiding damage caused by free radicals, which are implicated in a number of diseases as heart disease and oxidative stress (11).

A dietary soluble glycosylated phenol known as arbutin exhibits antioxidant (12, 13), anti-tumor (14, 15) and anti-inflammatory (16, 17). Arbutin can lessen oxidative stress by lowering the production of reactive oxygen species and superoxide since it has a long-lasting radical scavenging activity (18, 19). Arbutin has been extensively researched for usage as a hydroquinone substitute for skin whitening for almost 30 years now (20). Arbutin can be made in different ways, including chemical synthesis, hydroquinone bioconversion and plant extraction (21, 22). Arbutin can be produced by seven microbial enzymes, based on recent research, which includes cyclodextrin, alpha-amylase, sucrose isomerase, α-glucosidase, glycosyltransferase, dextransucrase and amylosucrase sucrose phosphorylase (23). A portion of the plant, developmental phases and harvest season all affect a plant's arbutin concentration (24, 25). The extraction and purification processes used affect the arbutin's production (26, 27). Arbutin was mentioned in previous studies, and it has the potential to prevent nerve damage and other disorders that result from nerve damage (28, 29). Also, found that arbutin promoted gut development, such as villus length villus areas (30). Furthermore, arbutin is utilized in therapy for Alzheimer's disease because of its extensive range of pharmacological effects (31). More benefits of this natural compound (arbutin) include the enhancement of wound recovery (32). It also provides radioprotection (33). The aim of the study is to improve the biological production of arbutin from a local isolate, improve the nutritional conditions for production to reach optimal conditions and prepare semi-industrial growing media using agricultural and food waste.

Materials and Methods

The bacterial isolates used were isolated and characterized by us; the source of the Arbutin standard chemical was (SIGMA), Spectrophotometer (Biotech engineering, England). Sucrose, maltose and glucose were obtained from Seelze-Hannover and molasse was obtained from the sugar factory (in Maysan / Iraq), which is considered an industrial waste from sugar cane. Nutrient Broth medium from (HiMEDIA).

Preparation of inoculum

Nutrient Broth (N.B) medium was prepared according to the manufacturer's instructions and sterilized by autoclave at 121 °C for 20 min; the medium was prepared in 10 mL tubes. Young cultures no more than 48 h old were ready from the isolates that were selected from the screening stage and were distinguished by their efficiency in producing arbutin. By activating them on the liquid nutrient N.B medium, taking a swab of the selected isolates using the metal loop carrier and immersing them in the liquid N.B medium, then incubating them at a temperature of 37 °C for 24 h. Finally, the absorbance was measured at the wavelength of 600 nm after zeroing the device with a blank control tube containing the liquid nutrient medium alone, N.B. Then the necessary dilution was performed to obtain the required number of cells that must be added to the arbutin production medium, using MacFarland equation.

Preparation medium arbutin production

Using the medium described in (34) with slight modifications, the submerged culture method was used to produce arbutin; the medium for arbutin production consisted of Nutrient Broth 13 g/L, 20 g/L of sucrose, 10 g/L of peptone, 0.5 g/L of $MgSO_4$, 1 g/L of K_2HPO_4, 1 g/L of KH_2PO_4, 2 g/L of NaCl and 1 g/L of $NaHCO_3$. Melt the ingredients mentioned above. They were distributed by 50 mL in flasks (250 mL), after which each flask was individually autoclaved, after inoculum with $1x10^6$ cell/mL from a bacterium (*Bacillus subtilis* NN2M) and incubated for 24 h at 37 °C. Following that, with the aseptic technique, the sterile hydroquinone (autoclaving) was added 5 mL of it to the production medium (0.5 % w/v).

After the end of the incubation period, the arbutin was extracted from the production medium by filtration through Whatman filter paper (No.1) with a vacuum. The filtrate was taken and put in a centrifuge at 3000 rpm at 4 °C for 10 min and the filtrate until use.

Preparation of the standard curve for estimation arbutin

A standard curve for arbutin determination was prepared according to the technique (35), with some modifications. A series of solutions were prepared by mixing 1 mL of of 2.5 mM potassium per iodate and arbutin in varying concentrations (25-150 µg/mL) and incubated in closed

tubes at room temperature for 20-25 min. Pipette 0.5 mL of the reaction mixture, add 7 mL borate buffer pH 8.0 and add 2 mL of freshly prepared 2 % solution of potassium iodide. The absorption spectra of the series of concentrations were scanned in the wavelength range of 300-500 nm. The absorbance of the resulting solutions was measured at 351 nm.

Optimal nutritional conditions for arbutin production

To determine the optimal nutritional conditions for the production of the glycoside phenolic compound from the local isolate of the bacteria (*Bacillus subtilis* NN2M), many factors affecting the output were studied, which included Carbon source, the concentration of optimum carbon source, nitrogen source, the concentration of optimum nitrogen source, different concentrations of ascorbic acid and biotin and the concentration of hydroquinone in production media.

Influence of carbon source

Different carbon sources (7 sources) were tested, including molasses, glucose, dried apple peel, dried potato peel, sucrose, maltose and dried pear peel at concentrations 2 % for all. The collected peels were washed with tap water and dried (hot air - oven) at 50 °C. Molasses was used as a carbon source after the process of purification for it. In addition, the effects of different concentrations of the optimal carbon source studied in the liquid production medium were 2, 4, 6, 8, 10 and 12 %.

Influence of nitrogen source: Yeast extract in the production medium was replaced by different nitrogen sources, including organic and inorganic sources at a concentration of 1 % (w/v), which included (8 sources) peptone, meat extract, casein, yeast extract, sodium nitrate, ammonium nitrate, ammonium sulfate and casamino acids. In addition, the effect of different concentrations of the optimal nitrogen source for production 0.2, 0.5, 1, 2, 4, 6, 8, 10 and 10 % (w/v) was also studied.

Influence of different concentrations of hydroquinone:

To determine the optimal concentration of hydroquinone for the production of arbutin in a liquid medium of production, different concentrations of hydroquinone (0.5, 1, 2, 3 and 4) % (w/v) were used to produce the bioactive compound (arbutin).

Statistical analysis

The Statistical Analysis System- SAS (2018) program was used to detect the effect of different groups on study parameters. The least significant difference-LSD test was used to compare the means in this study significantly (36).

Results

Influence of different carbon sources:

The results in Table 1 show that the production medium, which contained Molasses as a carbon source, was the best in the production of the bioactive compound (arbutin) by bacterium local isolate *Bacillus subtilis* NN2M during 24 h of incubation at 37 °C, in terms of concentration of 23.34 µg/mL followed by dried pear peel and sucrose with concentration of 18.98 µg/mL and 18.38 µg/mL respectively. There is a significant difference in the results of the molasses carbon source.

Table 1. Effect of Different carbon sources in the production of Arbutin from *Bacillus subtilis* NN2M

Carbon source	Absorbance at 350 nm	Concentration of bioactive compound (Arbutin)/ µg/mL
Molasses	0.3465	23.34 ± 1.51^{a}
Dried banana peels	0.1615	8.544 ± 0.64^{cd}
Glucose	0.268	17.06 ± 0.81^{b}
Dried apple peels	0.175	9.624 ± 0.67^{c}
Dried potato peels	0.12	5.264 ± 0.32^{d}
Sucrose	0.284	18.38 ± 0.75^{b}
Maltose	0.217	13.02 ± 0.63^{c}
Dried pear peels	0.292	18.98 ± 1.06^{b}
L.S.D. (P-value)	---	4.022 ** (0.0001)

** (P≤0.01).

Effect of carbon sources concentration:

Molasses was selected as an optimal carbon source for the production of the bioactive compound from *Bacillus subtilis* NN2M isolate during 24 h of incubation at 37 °C, In terms of concentration in the later experiments of this study. The results (Table 2) showed that the concentration at 4 % (w\v) was superior to the other concentrations for the production of the arbutin. Therefore, it was considered the best concentration of carbon source that can be added to the arbutin production media in terms of concentration of 27.62 µg/mL.

Table 2. Effect of carbon source concentration in the production of Arbutin from *Bacillus subtilis* NN2M

Concentrations of carbon source	Absorbance at 350 nm	Concentration of bioactive compound (Arbutin)/ µg/mL
2 %	0.308	20.26 ± 0.97^{b}
4 %	0.400	27.62 ± 1.61^{a}
6 %	0.228	13.90 ± 0.74^{d}
8 %	0.257	16.22 ± 0.59^{bcd}
10 %	0.2835	18.30 ± 0.84^{bc}
12 %	0.233	14.30 ± 0.52^{cd}
L.S.D. (P-value)	----	4.716 ** (0.0001)

** (P≤0.01).

Effect of different nitrogen sources

The results showed in Table 3 that the organic source of peptone exceeded the production of arbutin compared to other nitrogen sources, which had an essential effect on the increase in the production of a bioactive compound (arbutin). The resultant compound's concentration has risen from (27.62) to 47.3 µg/mL as shown in Table 3.

Table 3. Effect of different nitrogen sources in production of Arbutin from *Bacillus subtilis* NN2M

Nitrogen Sources	Absorbance at 350 nm	Concentration of bioactive compound (Arbutin)/ µg/mL
Meat extract	0.386	26.5 ± 1.07^{c}
Casin	0.392	26.9 ± 1.27^{c}
Yeast extract	0.48	34.02 ± 1.85^{b}
Peptone	0.602	43.7 ± 2.72^{a}
Casimino acid	0.474	33.5 ± 1.69^{b}
Sodium nitrate	0.248	15.4 ± 0.75^{d}
Ammonium nitrate	0.222	13.46 ± 0.58^{d}
L.S.D. (P-value)	----	6.481 ** (0.0001)
	** (P≤0.01)	

Effect of nitrogen sources concentrations: This study used different concentrations of organic nitrogen peptone to determine the optimum concentration of arbutin production. The results showed in Table 4 that the best nitrogen source was peptone and the optimum concentration was 2 % (w\v) because it recorded a higher compound concentration than any other concentration in terms of arbutin production. This peptone concentration was based on the arbutin production media in the later experiments of this study.

Table 4. Effect of nitrogen source concentrations in the production of Arbutin from *Bacillus subtilis* NN2M

Concentrations of nitrogen source	Absorbance at 350 nm	Concentration of bioactive compound (Arbutin)/ µg/mL
1 %	0.6395	46.78 ± 2.07^{b}
2 %	0.798	59.46 ± 2.75^{a}
4 %	0.3326	22.23 ± 1.06^{c}
6 %	0.287	18.62 ± 0.74^{c}
8 %	0.28	18.5 ± 0.87^{c}
10 %	0.176	9.7 ± 0.51^{d}
12 %	0.165	8.82 ± 0.47^{d}
With out	0.129	5.2 ± 0.28^{d}
L.S.D. (P-value)	---	6.944 ** (0.0001)
	** (P≤0.01)	

Effect of different concentrations of hydroquinone

This study used different concentrations of hydroquinone to determine the optimum concentration of arbutin production. The results are shown in Table 5. The maximum production of arbutin was 75.1 µg/mL at a concentration of hydroquinone (2 %) (w\v).

Table 5. Effect of different concentrations of hydroquinone in the production of Arbutin from *Bacillus subtilis* NN2M

Concentrations of hydroquinone	Absorbance at 350 nm	Concentration of bioactive compound (Arbutin)/ µg/mL
0.5 %	0.146	7.30 ± 0.41^{d}
1 %	0.788	58.66 ± 2.08^{b}
2 %	0.993	75.10 ± 3.15^{a}
3 %	0.254	15.90 ± 0.78^{c}
4 %	0.107	4.18 ± 0.26^{d}
L.S.D. (P-value)	---	8.169 ** (0.0001)
	** (P≤0.01)	

Discussion

Among the several isomers of the bioactive hydrophilic polyphenol is arbutin, beta-arbutin is the most common and prevalent one. Its production involves a wide range of natural and synthetic processes, including those involving various plant species, enzymatic reactions and bacteria that have had their metabolism altered. More and more research is focusing on arbutin biosynthesis (9). A total of seven microbial enzymes, including sucrose isomerase, cyclodextrin glycosyltransferase, alpha-amylase, amylosucrase sucrose phosphorylase, dextransucrase and α-glucoside, have been identified as capable of synthesis in recent lab investigations (37). Arbutin, a hydroquinone glycoside, serves a variety of biological purposes in pharmaceutical and cosmetic formulations (17). It is commonly used as an anti-ageing and skin-bleaching ingredient in cosmetic goods (23). Due to its numerous biological and pharmacological characteristics, as well as its distinct therapeutic advantages, arbutin has received particular interest (38, 39). Due to the plant's content of glycosides, such as glucosinolate, they have therapeutic applications (40). Turmeric and ginger powder are essential nutritive additives that are added to laying hens to improve product performance. The use of whole cells and microbial enzymes to produce arbutin has been the subject of several prior studies. Some microorganisms may convert hydroquinone into arbutin. For instance, in a previous study, 600 different strains of microorganisms that were recovered from soil were cultivated and checked for the ability to make arbutin (39). *Bacillus subtilis* X-23 enzyme-producing strain, *B. subtilis* X-23, was examined and extracellular amylase was shown to be the enzyme tested and extracellular amylase was shown to be the enzyme (19). Additionally, arbutin has been successfully produced by lyophilized *Xanthomonas campestris* cells (41).

Based on previous studies, we studied how arbutin is synthesized by using bacterial isolates from the soil and selecting the most efficient in production based on the quantitative estimation of the resulting compound, then

conducting microscopic and cultural examinations and genetic diagnosis depending on the 16S rRNA gene by us and selecting them for the rest of the study After genetic diagnosis, isolated bacterium was identified to be *Bacillus subtilis* NN2M which was used in the rest of the survey. The optimum nutritional factors for the arbutin biosynthesis were then investigated, including carbon source, the concentration of optimum carbon source, nitrogen source, the concentration of optimum nitrogen source and the concentration of hydroquinone in production media.

For arbutin biosynthesis, carbon sources are essential nutrients for the growth of microbial cells, and they serve as glucosyl donors. In a study conducted by (42), it was found that (*Xanthomonas campestris* TISTR 2065) can use maltose and sucrose as a substrate to synthesize arbutin and that the concentration of these disaccharides regulated the production yield of arbutin (32). They studied the effect of eight carbonate sources. The best carbon sources for cell reproduction were maltos. Furthermore, both maltose and sucrose were helpful in the production of arbutin (43); they studied the ability of Aspergillus flavus WJF81 to produce kojic acid and tried to improve its productivity. They found that sucrose 10 % is the best carbonic source at pH (4). Moreover, reports are on the usage of various cheap feedstock instead of pure sugar, such as muscovado, table sugar and sweet sorghum juice used in arbutin production (44). As mentioned in one report, the best yield of kojic acid from isolation *Aspergillus oryzae* using purified molasses as the optimal carbon source among a number of carbon sources tested (45).

Experiments were carried out using organic and inorganic sources by *Bacillus subtilis* NN2M in fermentation. The optimum nitrogen source was peptone (32). It was found that the best nitrogen source is in terms of product yield, and 3 peptone beef, fish and pork gave the best results. The nitrogen sources used are varied. In the production of nutrient media, the media containing the protein isolate was similar to the control medium (CzapekDox Agar) for growing molds. The source of nitrogen used in the arbutin production medium is one of the most important factors for building a number of organic compounds in bacterial cells, such as amino acids and proteins. The need for microorganisms may vary depending on the sources and the degree of their readiness for the bacterial cell (46).

The nutritional and environmental conditions of a bacterial isolate were studied and gallic acid was produced using agricultural and food waste as carbon sources (47). The highest productivity was using pomegranate peels at a concentration of 3 % and with a nitrogen source represented by ammonium chloride (48). In the process of biosynthesis, arbutin hydroquinone works as a glycosyl acceptor and in this study, the optimum hydroquinone concentration was 2 % (w/v). Meanwhile, (49) used a hydroquinone concentration of 10 (mM) (50) and declared the best concentration was 5 (mM).

Conclusion

In conclusion, the results demonstrated the possibility of producing the biologically active compound arbutin using waste from sugar refinery factories by means of local isolation of bacteria.

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Authors' contributions

Both authors, NMI and NMS, designed and performed the experiment, collected and analyzed the data, and prepared the manuscript. They isolated and characterized the bacterial isolate *Bacillus subtilis* NN2M, produced arbutin, and studied the optimal nutritional conditions for its production.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None.

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